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Spent grains – a new support for brewing yeast immobilisation

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Abstract

A novel carrier obtained from spent grains, a brewing by-product, was used for brewing yeast immobilisation in a continuous bubble-column reactor. The multiple-layer cell adhesion to the carrier particles resulted in a maximum cell load of 430 mg dry cell g^{-1} dry carrier (d.c.). After 120 h of reactor operation, the cell load of DEAE-modified carrier was below 40 mg dry cell g^{-1} d.c. while the values for non-modified carrier reached at least 100 mg dry cell g^{-1} d.c. The changes in substrate composition on the rate of yeast attachment and on its stability were also studied.

Introduction

The conventional brewing process has an extremely long history and can be regarded as a typical example of traditional biotechnology. However, several contemporary engineering achievements and scientific inventions have already found application in the brewing process, including continuous bioreactors with immobilised yeast. Continuous beer production is promising mainly due to the high potential for savings in production time, investments and production costs compared with traditional brewing methods. Therefore, reactors for primary and secondary fermentation with immobilised yeasts have been intensively studied over the last three decades (Masschelein 1997). However, there are a number of technical difficulties including flavour problems, contamination, yeast viability, removal of excess yeasts and CO_2, process optimisation, channelling and clogging of the reactor, carrier cost and regeneration. The carrier cost is often restrictive in terms of the economic feasibility (Mensour *et al.* 1997) and, besides the carriers available at the present moment (cellulose derivatives, porous glass, silicon carbide and wood chips), there is a need for cheap support material with advantageous properties: convenient immobilisation, high cell loading, low mass transfer limitations, stability, rigidity, possibility to regenerate and sterilise, flexibility for reactor designs, food grade (Linko *et al.* 1998). Taking into account these requirements and trying to meet the low price target, 'spent grains', a brewing by-product with considerable cellulose content, was studied as a potential carrier for yeast immobilisation.

The aim of this work was to develop a simple method to obtain a cellulose based carrier from spent grains, to develop a method for determination of the immobilised biomass, to verify the carrier's suitability for yeast immobilisation and to compare it with its surface modified with diethylaminoethyl (DEAE).

Materials and methods

Microorganism and media

An industrial yeast strain of *Saccharomyces cerevisiae* from a brewing company (Unicer, SA) was cultivated in 100 ml medium under aerobic conditions on a rotary shaker (150 rpm) at 30 °C for 48 h. The composition of the medium was as follows (g l^{-1}): KH_2PO_4, 5; $(NH_4)_2SO_4$, 2; $MgSO_4 \cdot 7H_2O$, 0.4; yeast extract, 1; glucose, 10. In continuous culture experiments a medium with the same composition was used with

glucose content of 10 or 100 g l^{-1}. Sterilised wort supplied by Unicer, SA was also used as medium.

Continuous experiments

The yeast immobilisation experiments were carried out in a continuous bubble column reactor with total working volume of 440 ml and a height to diameter ratio of 1.5. The carrier (6–7 g dry wt) was placed in the reactor with the medium described above (with 10 g glucose l^{-1}) and inoculated with 100 ml pre-cultured brewing yeast suspension. The continuous feed started after 16 h of batch growth. The medium was supplied at the bottom of the reactor by means of a peristaltic pump. Sterile air was passed into the bubble column at 0.9 l h^{-1}, through a pipe with 4 holes (1 mm diameter) placed at the bottom of the reactor. The reactor was operated at various dilution rates from 0.2 to 0.8 h^{-1}. The reactor outlet was connected to an overflow.

Preparation of the carrier

Dry spent grains (100 g) were mixed in 1500 ml of 0.35 M HCl solution at 60 °C for 2.5 h to hydrolyse the residual starchy endosperm and embryo of the barley kernel present in the spent grains. The mixture was cooled, washed with water and dried. The remaining solids (ca. 30 g), mainly the husks of the barley grain, were partially delignified by shaking (120 rpm) in 500 ml of 0.5 M NaOH at 30 °C for 24 h. After being washed several times with water until neutral pH and dried, the carrier (ca. 10 g) was ready to be used. The preparation procedure gives 10% (w/w) yield from dry spent grains. The drying steps applied in the preparation procedure were necessary only in order to quantify the yields.

Chemical modification of the carrier

The preparation of the diethylaminoethyl-modified carrier (DEAE-cellulose) was carried out as follows. The cellulose-based carrier (7 g) was immersed into 200 ml distilled water and 60 g Na_2SO_4 and 8.5 g NaOH were added. The mixture was heated to 40 °C following which 10 to 20 g of 50% (w/v) diethylaminoethylchloride aqueous solution (depending on desired degree of derivatisation) was added over 2 h with continuous stirring. The reaction mixture was then heated to 60 °C for approximately 30 min, then cooled, washed with distilled water and dried at 60 °C (Antrim & Harris 1991).

Immobilised biomass determination

A sample containing 0.1 to 0.2 g dry carrier was taken from the reactor. The loosely attached cells were removed by gentle mixing (2 min, 200 rpm) of the carrier in 20 ml 1.5 g NaCl l^{-1}, pH 3.0 and washed once with 20 ml of the same solution.

The more strongly attached biomass was removed by vigorous mixing (600 rpm) of the carrier in 20 ml of 1.5 g NaCl l^{-1} for 30 min. The amount of released biomass was determined after settling the carrier by measuring the turbidity (A) of the bulk liquid at 620 nm and was correlated to cell dry weight by a calibration curve. The whole procedure of mechanical cell removal was repeated while the bulk liquid contained a significant amount of released cells ($A_{620} > 0.1$).

The residual amount of cells firmly attached to the carrier and resistant to mechanical detachment was determined by protein measurement. This method was based on extraction of cell proteins followed by a quantitative assay. The proteins were extracted by boiling the carrier with cells in 10 ml 1 M NaOH for 10 min. The alkaline mixture was cooled and neutralised by 10 ml 1 M HCl. The protein concentration in the mixture was determined by the Bradford method and correlated to the cell concentration by a calibration curve. It was verified that there was no release of proteins from the pure carrier after boiling in 1 M NaOH for 10 min.

Analytical methods

Ethanol concentration was measured by HPLC using a column (Polyspher CH CA, Merck), a refractive-index detector (Jasco 830-RI) and a pump (Jasco 880-PU). The eluent used was 0.005 M H_2SO_4, at a flow rate of 0.75 ml min^{-1} being the column at a temperature of 60 °C. Glucose was measured by the DNS method. The ion exchange capacity, defined as a number of equivalents of exchangeable ions per weight of exchanger (meq g^{-1}) of the anion exchanger DEAE-cellulose, was determined by titration of the charged groups with 0.1 M NaOH solution. Average surface area of particles (S) was determined using an image analysis procedure (Vicente *et al.* 1996). Three particle sizes were used $S_1 = 0.59$ mm^2, $S_2 = 0.247$ mm^2 and $S_3 = 0.105$ mm^2.

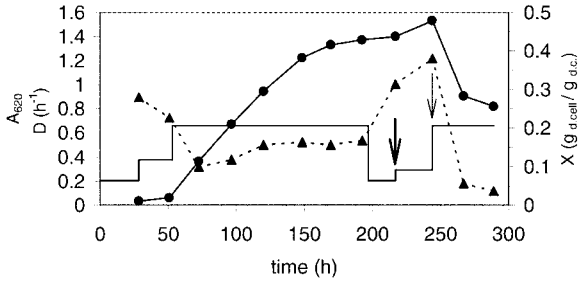

Fig. 1. Development of yeast biofilm in terms of cell load X (●, g dry cell g^{-1} dry carrier) on the surface of the carrier ($S_3 = 0.105$ mm^2) during continuous experiment. A_{620} (▲, free biomass at 620 nm); D (— dilution rate, h^{-1}); ➔ change from synthetic medium to wort ($t = 217$ h); → change from wort to distilled water ($t = 245$ h).

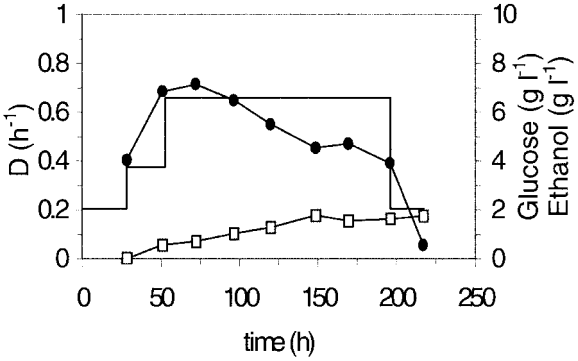

Fig. 2. Glucose (●) and ethanol (□) concentrations during the biomass attachment as shown in Figure 1.

Results and discussion

We assume that the onset of the massive yeast attachment on the surface of our carrier can be considered as a result of continuous culture induced physiological changes of the yeast cells, particularly of their surface properties. This hypothesis is also supported by the fact that in batch experiments, where the growth conditions are considerably different, there was no spontaneous cell adhesion observed.

The continuous experiments were carried out at a high dilution rate in order to decrease the number of free cells in the reactor and favouring thus the growth of the attached cells colonising the surface of the carrier. Between 50 and 100 h of continuous reactor operation a sudden increase in the cell attachment rate was observed and it continued until a balanced steady state between the attached and outgrowing cells was established (Figure 1). The concentration of free biomass in the reactor (A_{620}), was either following the changes in dilution rate (D) or feed composition. High D values and starvation (distilled water) caused depletion of free cells in the reactor, while a shift-down in D or increasing substrate concentration (wort) resulted in free cell accumulation (Figure 1).

In the course of the experiments carried out in the bubble column reactor at high dilution rates (D), only a partial consumption of the inlet glucose (10 g l^{-1}) has been observed (Figure 2). However, when the dilution rate was decreased to $D = 0.2$ h^{-1} at the stage of the maximum cell load (X) ($t = 196$ h, $X = 430$ mg dry cell g^{-1} dry carrier) the glucose consumption was near complete. After the beginning of the intensive cell attachment ($t = 50$ h) ethanol was also present in the reactor outflow in concentrations up to 2 g l^{-1} (Figure 2) confirming thus the oxidoreductive glucose consumption by the yeast cells.

The microscopic observations revealed that the yeast cell adhesion to the surface of the plain sheet-like carrier particles occurred in multiple layers (Figure 3). The thickness of the biofilm several times surpassed the thickness of the carrier particles significantly increasing the initial volume of the carrier material in the reactor.

In order to shorten the time required to initiate the intensive yeast accumulation and increase the yeast immobilisation rate, the effect of the chemical modification of cellulose carrier was examined. Experiments with chemically modified and non-modified carriers were carried out under the same conditions. We assumed that the positively charged DEAE ion-exchange group could favourably effect the attachment of the negatively charged yeast cells (Zeta potential −10.5 mV) under experimental conditions. In fact, the DEAE groups slightly increased the initial cell load, up to 18 mg dry cell g^{-1} dry carrier (mg dry cell g^{-1} d.c.), comparing to the non-modified carrier of the same size (S_1). Nevertheless, the chemical modification of the carrier rather delayed the beginning of the intensive yeast attachment (Figure 4). In the course of the experiment the DEAE modified carrier turned from its typically light yellow colour to dark brown. This can be explained by the attachment of other negatively charged components of the medium (products of Maillard reaction, proteins) competing with yeast cells and so negatively influencing their adhesion.

The start of the rapid yeast attachment, its rate and extent were influenced by the carrier particle size. The decrease of the average particle area, from S_1 to S_3, speeded up the biofilm formation rate and the maximum cell load, probably by increasing the number

Table 1. Cell load in different continuous fermentation systems.

Application and carrier	Cell load ([a] or [b])	Reference
Continuous beer fermentation, Ca-alginate	80[a]	Shindo *et al.* (1994)
Continuous beer fermentation, DEAE-cellulose	3.2[b]	Andersen *et al.* (1999)
Secondary fermentation, DEAE-cellulose	40[a]	Grönquist *et al.* (1989)
Primary fermentation, DEAE-cellulose	8[a]	Kronlöf *et al.* (1989)
Primary fermentation, kappa-carrageenan	32[b]	Mensour *et al.* (1997)
Secondary fermentation, Ca-alginate, Ca-pectate, kappa-carrageenan	50[a]	Dömény *et al.* (1998)
Primary fermentation, Ca-alginate, Ca-pectate	50[a]	Šmogrovicová *et al.* (1997)
Primary fermentation, silicon carbide matrix	15[b]	Andries *et al.* (1997)
Ethanol production, beech wood chips	150[a]	Razmovski *et al.* (1996)
Glucose fermentation, spent grains	430[a], 6[b], 21[b]*	This work

[a]mg dry cell g^{-1} d.c.
[b]mg dry cell ml^{-1} reactor volume.
*Theoretical value (see text).

of active sites for initial cell attachment as well as the surface area to weight ratio of the carrier. It resulted in maximum cell load values of 268, 364 and 430 mg dry cell g^{-1} d.c. for the average particle area of the carrier $S_1 = 0.59$ mm^2, $S_2 = 0.247$ mm^2 and $S_3 = 0.105$ mm^2, respectively (Figure 5).

The maximum cell load values for our carrier are significantly higher than those reported in previous studies on continuous brewing with immobilised cells (Table 1). As can be seen, the cell load range for DEAE-cellulose is between 8 and 40 mg dry cell g^{-1} d.c., which corresponds to yeast monolayers and is in agreement with values found for DEAE modified carrier studied in our work (18 mg dry cell g^{-1} d.c.).

Cell loads expressed as a number of cells per carrier weight (volume of reactor) given by other authors were converted into mg dry cell g^{-1} dry carrier (ml rector volume) considering the diameter of the brewing yeasts 8 μm, their water content 70% (v/v) and density close to water (Alberts *et al.* 1994). The yeast cell load value closest to those in the present work was found for beech wood chips as a carrier for ethanol fermentation (150 mg dry cell g^{-1} d.c.) where authors also referred about multilayer immobilisation (Razmovski & Pejin 1996). The higher cell load presented in our work can be ascribed to the significantly lower average size range of our carriers (0.8–0.3 mm) than was the size of the wood chips (1.84 mm). Comparing to the previously published biomass contents per volume of reactor our study (applying 1.4% w/v initial carrier volume fraction) showed a relatively low value of 6 mg dry cell ml^{-1} reactor volume. However, by increasing theoretically the initial carrier volume fraction to 5% (w/v) the biomass concentration in the reactor would be around 21 mg dry cell ml^{-1} reactor volume which is close to the highest values published so far (Table 1).

The influence of starvation, pH-shift and increased substrate concentration on the stability of the yeast biofilm has been studied as well. A sudden pH stress provoked by decreasing the synthetic medium pH from 5 to 2.5 stopped during 24 h the cell accumulation on the carrier particles. Starvation of the biomass in synthetic medium without carbon and energy source resulted in 10% decrease of the immobilised biomass within 24 h (data not shown). The combined effect of starvation, pH and osmotic stress enhanced by the increase of D from 0.3 h^{-1} to 0.66 h^{-1} caused by change from wort (pH 5) to distilled water (pH 2) (Figure 1; $t = 245$ h) resulted in almost 50% decrease of cell load during 48 h. However, this cell release could be used later for removal of excess biomass, yeast regeneration or reduction of undesirable bacterial contamination. The stability of the attached cells caused that not even a repeated vigorous mixing resulted in complete biomass removal thus requiring the additional biomass determination through protein measurement.

The change in glucose concentration, e.g., from 10 g l^{-1} to 100 g l^{-1} (data not shown) or a change to wort (ca. 150 g reducing sugars l^{-1}) had no negative effect on the developed yeast biofilm. On the contrary, the real wort fermentation increased the cell load up to 480 mg dry cell g^{-1} d.c. in the case of S_3 (Figure 1; t from 217 to 245 h). Despite of the low carrier volume fraction (1.4% w/v) used during the wort

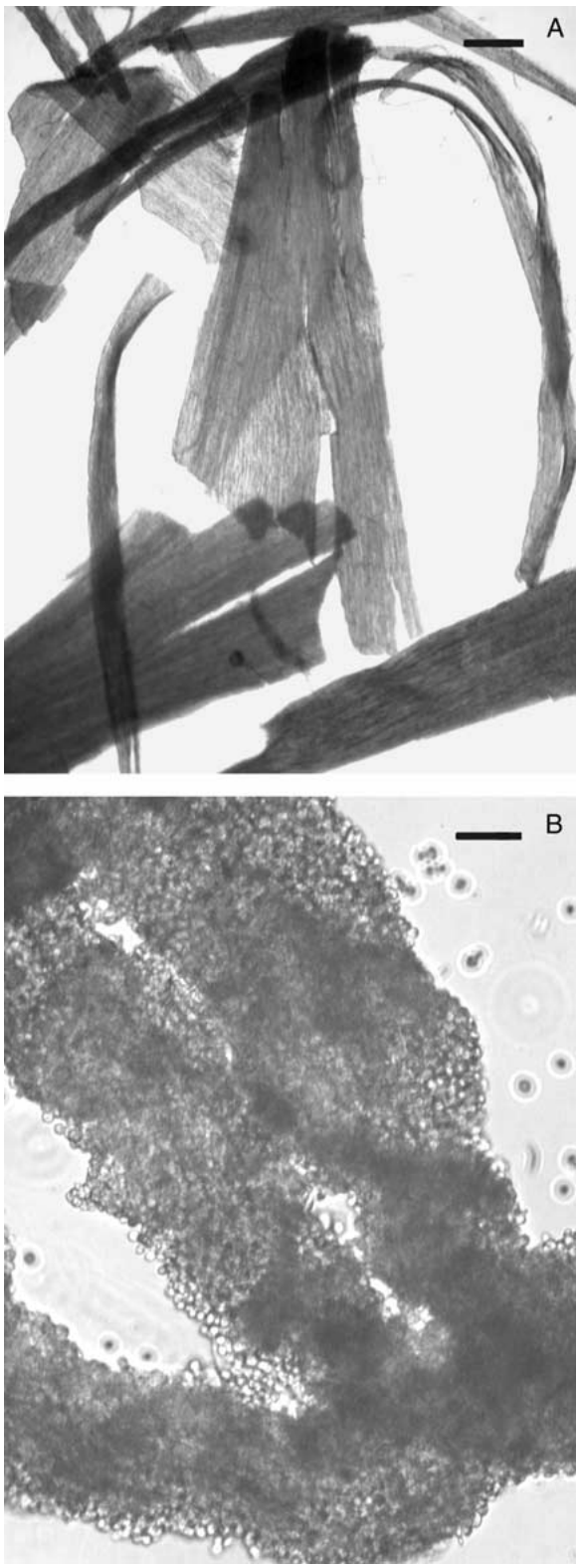

Fig. 3. (A) Photograph of the sheet-like carrier obtained from spent grains; (B) photograph of the carrier with attached yeast cells. Bars correspond to 0.1 mm.

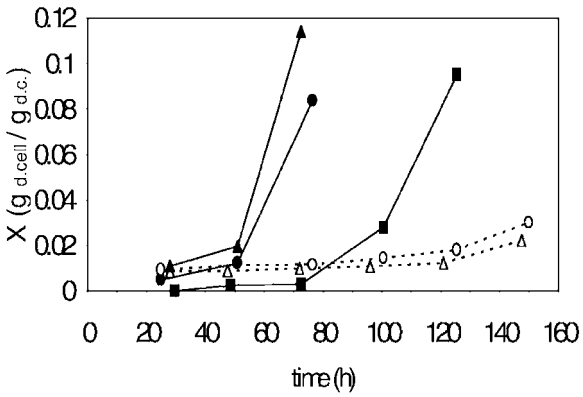

Fig. 4. Comparison of the initial yeast attachment on non-modified carrier of different average particle area S_{1-3} (■, $S_1 = 0.59$ mm^2; ●, $S_2 = 0.247$ mm^2; ▲, $S_3 = 0.105$ mm^2) and DEAE modified carrier of the size S_1 with two different ion exchange capacities (△, 0.45 meq g^{-1}; ○, 0.80 meq g^{-1}).

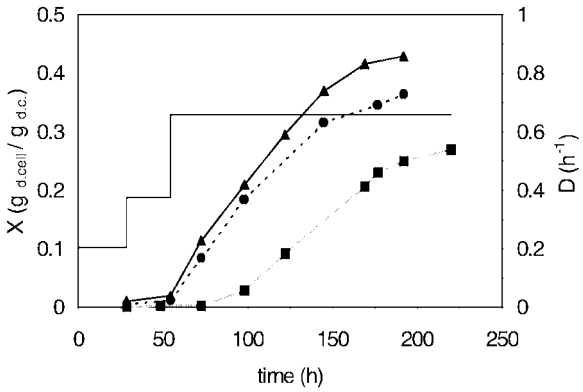

Fig. 5. Development of the maximum cell load (X) in dependence on average particle area (S) during continuous experiments: ■, $S_1 = 0.59$ mm^2; ●, $S_2 = 0.247$ mm^2; ▲, $S_3 = 0.105$ mm^2; —, D (h^{-1}).

fermentation the process resulted in an attenuation of 20% and ethanol content of 1.75% (w/v) obtained at $D = 0.2$ h^{-1}. We believe that further optimisation of the aeration rate (air or CO_2), dilution rate, bioreactor design and carrier volume fraction will significantly improve the process performance.

Conclusions

In the whole range of dilution rates and carrier particle sizes the ratio between the biomass immobilised on the carrier and the free biomass in the reactor is between 1.5 ($D = 0.2$ h^{-1}, S_1) and 12.3 ($D = 0.66$ h^{-1}, S_3). This is significantly higher than the range 0.7–2.3 published elsewhere (Tata *et al.* 1999).

The isolation yield of the clean carrier from dry spent grain is 10% (w/w). At this point it should be stressed that the carrier preparation procedure described in this article was performed in order to obtain a pure support not releasing proteins interfering with the biomass determination. This carrier preparation could be considered slightly labour demanding, however, it is counterbalanced with the reusability of the carrier after washing in caustic. For industrial applications the carrier preparation can be further simplified consisting only of washing in caustic.

These results suggest that spent grains are a promising alternative to the available carriers for continuous beer fermentation, the advantages being as follows: high yeast loading capacity, easy preparation (not requiring chemical modification), regeneration by simple washing in caustic, inert under fermentation conditions and the fact of being a brewery by-product not requiring investments.

Further experiments with continuous beer fermentations are going to be carried out in order to study the metabolic behaviour of the attached yeast cells, using an airlift bioreactor as it has been shown to be a good alternative to the packed or fluidised bed bioreactors used in other studies (Vicente *et al.* 1999).

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